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Prevalence of Genes Involved in Colistin Resistance in *Acinetobacter baumannii*: First Report from Iraq

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Microbial Drug Resistance

Mary Ann Liebert Inc

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Prevalence of genes involved in Colistin Resistance in <i>Acinetobacter baumannii</i>:	1
First report from Iraq	2
Running title: Colistin Resistance in <i>Acinetobacter baumannii</i>	3
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Manuscript writing and proof	23
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Abstract 30

Background and aim: Colistin is increasingly being used as a ‘last-line’ therapy to 31
treat infections caused by multi-drug resistant *Acinetobacter baumannii* (*A. baumannii*) 32
isolates, when essentially no other options are available in these days. The aim of this 33
study was to detect genes associated with Colistin resistance in *A. baumannii*. 34

Methods: 121 isolates of *A. baumannii* were collected from clinical and environmental 35
samples during 2016 to 2018 in Baghdad. Isolates were diagnosed as *A. baumannii* by 36
using morphological tests, Vitek-2 system, 16SrRNA PCR amplification and 37
sequencing. Antibiotic susceptibility test was carried out using disc diffusion method. 38
Phenotypic detection of colistin resistance was performed by CHROMagarTM COL- 39
APSE medium and broth microdilution method for the determination of the minimal 40
inhibitory concentration (MIC). Molecular detection of genes responsible for colistin 41
resistance in *A. baumannii* was performed by PCR. 42

Results: 92 (76%) out of 121 *A. baumannii* isolates were colistin resistant. 26 (21.5%) 43
out of 121 isolates showed positive growth on CHROM agar *Acinetobacter* base for 44
MDR. PCR detected *mcr-1*, *mcr-2* and *mcr-3* genes in 89 (73.5%), 78 (64.5%) and 82 45
(67.8%) in the *A. baumannii* isolates respectively. 78 (64.5%) out of 121 isolates 46
harbored the integron *intI2* gene and 81 (66.9%) contained *intI3* gene. Moreover, 60 47
(49.6 %) out of 121 isolates were positive for the quorum sensing *Iasl* gene 48

Conclusion: The presence of a large percentage of colistin resistant *A. baumannii* 49
strains in Baghdad may be due to the presence of mobile genetic elements and it is 50
urgent to avoid unnecessary clinical use of colistin. 51

Keyword: Colistin, Resistance, *Acinetobacter*, CHROMagarTM COL-APSE, pEtN 52
gene, CMS, mobilized colistin resistance. 53

Introduction 54 55

Colistin is a polymyxin E, which possesses cyclic deca-peptide linked to a fatty acyl 56
chain by α -amide linkage. The only difference in structure between polymyxin E and 57
B is a single amino acid ¹. There are two forms of colistin that are commercially 58
available for use : colistin sulfate and sodium colistin methanesulfonate (CMS) ². In the 59

1970s, CMS was replaced by aminoglycosides because of the significant side effects of these antibiotics such as nephrotoxicity and neurotoxicity ³.

Colistin is an antibiotic that is significantly used against Gram-negative bacteria ⁴⁻⁵. Due to increased and sometimes-inappropriate use, a rise in colistin resistance was reported ⁶. The bacterial cell membrane can be disrupted by polymyxins, which interfere with phospholipids leading to damage to the osmotic barrier ⁷. Polymyxins are polypeptide molecule with positive charge that act as antimicrobial by disrupting the cell membrane and leading to death of the cell. This disruption occurs as a result of polymyxins binding with negatively charge in lipid A moiety of lipopolysaccharides (LPS) ⁸. Resistance to colistin might occur by alteration in binding site in lipid A or efflux pumps ⁹.

The modification of lipopolysaccharide (LPS) is most prevalent method of resistance, which involves an addition of phosphoethanolamine (PEtN) groups. This is thought to alter the physical properties of the outer membrane, which leads to polymyxin resistance ⁷. There are many well-known of PEtN transferases for example EptA from *E. coli*, *H. pylori* and *Vibrio cholerae*. Another example is *PmrC* from *A. baumannii*. These enzymes are chromosomally encoded and catalyze the transfer of PEtN from phosphatidylethanolamine (PE) onto the lipid A moiety of LPS⁸.

Plasmid-mediated colistin (COL) resistance due to the Mobilized colistin resistance *mcr-1 pEtN* gene has recently been identified in Asian countries. Bacteria carrying the *mcr-1* gene have been isolated from many clinical and environmental sources since it was first described. Moreover, these isolates are often pan-drug resistant (PDR was known as non-susceptibility to all agents in all antimicrobial categories) or extensively drug resistant (XDR was known as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories), bacterial isolates remain susceptible to only one or two categories), which significantly limits the therapeutic options for those organisms ¹⁰. Many studies have identified seven *mcr* gene families in addition to *mcr-1*: *mcr-2* ¹¹, *mcr-3* ¹², *mcr-4* ¹³, *mcr-5* ¹⁴, *mcr-6* ¹⁵, *mcr-7* ¹⁶ and *mcr-8* ¹⁷.

Acinetobacter baumannii (*A. baumannii*) is a multidrug resistant (MDR) bacteria that can spread to civilian hospitals by cross infection of injured military patients repatriated from war zones ¹⁸⁻¹⁹. Two different mechanisms of colistin resistance have been characterized in *A. baumannii* ²⁰⁻²¹. The first mechanism includes complete inactivation of the lipid A biosynthetic pathway and loss of outer membrane LPS. This pathway could be inactivated by deletions, point mutations or insertions in any of three

genes (*lpxA*, *lpxC*, and *lpxD*)²². Consequently, the interaction between LPS and colistin can be prevented leading to increase the MICs of colistin. Colistin resistance due to LPS inactivation has been identified in laboratory mutants and recent clinical isolates²³. The second mechanism of colistin resistance is mediated by the PmrAB two-component system²⁴⁻²⁵. It has been shown that mutations in *pmrB* increased cell sensitivity to colistin more than 100 fold²⁴.

Integrans are genetic elements that allow efficient capture and expression of exogenous genes that may lead to dissemination of antibiotic resistance, particularly among Gram-negative bacteria^{13, 16}. Integrans are reported to play a main role in the distribution of colistin resistance²⁴⁻²⁵.

Antibiotic resistant bacteria communicate through quorum sensing (QS). Quorum sensing system is widely spread in bacteria, which possesses an important role in controlling virulence factors. Therefore, it is considered as a “speaking” system in bacterium²⁶. QS is a way bacteria secret chemical signals called auto-inducers to communicates with each other and is often followed by alteration in expression in genes expression^{3, 10, 16}. The persistent modification of bacterial species or strains is a global issue. Both gram-positive and gram-negative bacteria uses inducer called acylated homoserine lactones (AHLs) as a chemical signal or auto chemo-inducer. Although the mechanisms of signaling are different from species to species^{3, 10, 16}. QS-controlled gene expression plays a major role in the antibiotic resistant in pathogens.

The aim of this study was to detect genes, which might be associated with colistin resistance in *A. baumannii*. As a result of the increasing distribution of serious infections with gram negative bacteria, colistin is increasingly being used as therapy to treat infections caused by MDR *A. baumannii* because there is a lack of other options.

Material and Methods

This work was done as a collaboration between Mustansiriyah University, Iraq, Assiut University, Egypt and University of Cincinnati medical center, USA.

In this study, 121 isolates of *A. baumannii* were collected from clinical (30 isolates from urine samples, 47 isolates from blood, 31 isolates from wound swabs, 4 isolates from cerebrospinal fluid and one isolate from endotracheal tube) and environmental (

8 isolates from soil) samples from different hospitals across Baghdad during 2016 - 2018.	126 127
Detection of <i>A. baumannii</i>:	128
The phenotypic characterization was performed using morphological tests, CHROMagar <i>Acinetobacter</i> , and Vitek-2 system (BioMérieux, France).	129 130
Conventional PCR was performed for the genotypic identification of <i>A. baumannii</i> species using specific primers for <i>16SrRNA</i> gene as previously described. ²⁷	131 132
The sequence of the primers and PCR cycling conditions are listed in Table (1).	133 134
Phenotypic detection of colistin resistance in <i>A. baumannii</i>:	135
We used CHROMagar™ COL-APSE (Paris, France) media for detection of colistin resistance and broth microdilution method for the determination of minimal inhibitory concentrations (MIC). Broth microdilution is recommended by CLSI for testing colistin susceptibility. Strains which showed colistin MIC values >2 µg/mL were interpreted as resistant according to CLSI, 2016 breakpoints ⁽²⁴⁾ , and using quality controlled standard strains (<i>Acinetobacter baumannii</i> ATCC BAA-747) obtained from American Type Culture Collection.	136 137 138 139 140 141 142 143
Phenotypic detection of MDR in <i>A. baumannii</i>	144
Then used CHROMagar <i>Acinetobacter</i> Base with supplement (S) and MDR Supplement for detection on MDR isolates (MDR: resistance to C3G, quinolones, carbapenem etc). We prepared CHROMagar™ COL-APSE plates using dehydrated CHROMagar™ base media (X207B) with the CHROMagar™ COL-APSE supplement (X207S) + CHROMagar™ Anti-swarmer supplement (X208). These mediums were not autoclaved in order preserve the CHROMogenic compounds included in the mixture and instead were sterilized by boiling at 100°C while swirling or stirring regularly, prior to the addition of the supplements.	145 146 147 148 149 150 151 152 153
The antibiotic susceptibility profile for <i>A. baumannii</i> isolates was determined using Kirby-Bauer disc diffusion test and interpreted as recommended by Clinical Laboratory Standards Institute ⁽²⁴⁾ . Susceptibility testing was performed by inoculating Mueller-Hinton agar plates (Thermo Fisher Scientific and Waltham, MA, USA) used the suspension equivalent in turbidity to 0.5 McFarland. Then, we incubated the plates overnight at 37°C before recording the results.	154 155 156 157 158 159

	160
The following commonly used antibiotics were tested: ampicillin, amoxycillin,	161
aztreonam, cefepime, cefotaxime, cefoperazone, ceftazidime, imipenem, meropenem,	162
clindamycin, colistin, gentamicin, amikacin, tetracycline, chloramphenicol,	163
ciprofloxacin, amoxycillin/clavulanic acid and trimethoprim/sulphamethoxazole.	164
	165
Molecular detection of Colistin resistance genes:	166
The entire genomic DNA extraction was performed for all resistance isolates	167
according to modified Microwave lysis method ²⁵ .	168
Colistin resistance genes <i>mcr-1</i> , <i>mcr-2</i> and <i>mcr-3</i> were detected by PCR for all isolates	169
grown on CHROMagar TM COL-APSE.	170
The primer sequences and the amplicon size of different genes are listed in Table (1).	171
Briefly, the PCR reaction mixture consisted of 12.5 µl of 2X GoTaq®Green Master	172
Mix (KAPA, South Africa), 3 µl template DNA, 2 µl primers for each forward and	173
reverse primers with final concentration (0.6 pmol/ µl), and complete the volume to 25	174
µl with nuclease free water. The amplified PCR product was run in agarose gel	175
electrophoresis and compared with 100 bp DNA ladder (KAPA, South Africa) and then	176
visualized under UV trans-illuminator.	177
	178
Detection of integrons on colistin resistant <i>A. baumannii</i>:	179
PCR was used to detect <i>intI2</i> and <i>intI3</i> genes, which represent the class 2 and class 3	180
integrons that are known to be associated with MDR <i>A. baumannii</i> . The primer	181
sequences and the amplicon size of genes are listed in Table (1).	182
	183
Detection of quorum sensing in colistin resistant <i>A. baumannii</i>:	184
The presences of <i>Iasl</i> gene, as a part of the QS system, was investigated by PCR in <i>A.</i>	185
<i>baumannii</i> isolates. The primer sequences and the amplicon size of different genes are	186
listed in Table (1).	187
<u>Results and Discussion</u>	188
-Phenotypic properties	189
121 isolates of <i>A. baumannii</i> were collected from different samples during 2016 to	190
2018 in Baghdad. <i>16SrRNA</i> PCR amplification and sequencing were used to identify	191

A. baumannii isolates. *A. baumannii* is gram negative, can cause many infections due to its multi-drug resistance^(18, 20, 25).

26 (21.5%) out of 121 isolates showed positive growth with red colonies on CHROM agar *Acinetobacter* base for MDR suggesting the isolates were resistant to C3G, quinolones and carbapenem (CHROMagar™ *Acinetobacter*, 2018). The source of these 26 MDR isolates was environmental sample (n=1), from urinary tract infections (n=2), blood (n=12), wound swabs (n=6), cerebrospinal fluid (n=4) and endotracheal tube (n=1)

- Resistance of *Acinetobacter*:

The ability of the isolates to resist colistin was investigated by using CHROMagar™ COL-APSE (Paris, France) medium and confirmed by broth microdilution for determination of MIC. Isolates were detected by cream colonies on this medium (CHROMagar™ COL-APSE, 2018). The results showed that 92(76.03%) isolates (including one environmental isolate) showed positive growth on the CHROMagar™ COL-APSE medium. In addition, the MIC for these isolate was measured using broth microdilution for determination of MIC. The isolates showed MIC value ranged from 4 to 16 g/ml.

Antibiotic susceptibility pattern of *A. baumannii* isolated from clinical and environmental samples for different classes of antibiotics showed in Table (2). The result showed that the isolates were 100% resistant to β -lactum and Cefotaxime antibiotics, while they were less resistant (30%) to Tetracycline. PCR were performed to investigate the *mcr* related genes and their role in the resistance of *A. baumannii*

The *mcr*-1 gene was detected in 89 (73.6%) isolates; the *mcr*-2 gene detected in 78 (64.5%) and the *mcr*-3 gene was detected in 82 (67.7%) in the *A. baumannii* (Table 3).

CHROMagar COL-APSE medium was able to support the growth of colistin resistant Gram-negative bacteria because it is a sensitive and specific media for the growth of colistin resistant bacterial pathogens with a lower limit of detection of 10¹ CFU²⁶. Resistance to antibiotic is a global issue. The limitation of effective treatment carbapenem treatment is leading to reduce treatment options for multidrug resistant bacteria²⁸⁻³⁴. The (mobilized colistin resistance) *mcr*-1 gene has been reported in *Escherichia coli* and *Klebsella pneumoniae* from China, which encodes phosphoethanolamine transferase¹⁰. It has the ability to be transferred between different

bacterial strains. This leads to antibiotic resistance because of alterations in the bacterial cell membrane lipid A ^{10, 23, 35}. Gene *mcr-2* has been reported in 76% of bacteria with *mcr-1* gene from Belgium ¹¹. In addition, *mcr-3* gene has been recently reported in *E. coli* of pig origin, which showed a 45.0% and 47.0% identity in nucleotide sequence to *mcr-1* and *mcr-2*, respectively ¹².

Results highlighted the rapid spreading of *mcr-1*, *mcr-2* and *mcr-3* genes globally. Recently, *mcr-1* has been isolated from *Enterobacteriaceae* (animals), products of animals, humans and environments in more than thirty different countries from five continents ³⁶. This rapid increase in the reporting of resistance mechanism in a short time is alarming.

- Role of integrons on MDR distribution

PCR was used to detect the class 2 and class 3 integrons in isolates of *Acinetobacter baumannii*, which associated with multi-drugs resistances. The results showed out of 121 isolates, 78 (64.5%) harbored *intI2* gene and 81 (66.9%) contained *intI3* gene (Table 3). These results confirmed the role of integrons in MDS distribution in *A. baumannii*, which is similar to the role of integrons in the distribution of MDR in *Salmonella* spp. in Rajaei, et al. ³⁷.

Integron genes play a key role in the horizontal transfer of antibiotic multi-resistance accompanying with genetic element. Resistance genes are either on the host plasmid or bacterial chromosome ^{13, 38-39}. The *intI* gene encodes for an integrase, which belongs to the tyrosine-recombinase family ⁴⁰. The activity of integrase includes recombination of separate DNA molecules as gene cassettes. Integrons are divided into two subsets: the mobile integrons that are responsible for spreading the anti-drug resistance genes and super integrons. According to sequencing, there are five classes of integrons ⁴¹⁻⁴².

Integrons have the ability to capture the antibiotic resistance cassettes genes that lead to distribution of MDR and decrease the infection treatment options ⁴³. Resistance cassettes have been reported in both gram negative bacteria and gram positive bacteria ⁴⁴⁻⁴⁶.

- Quorum sensing detection in *Acinetobacter*

This system was first discovered in 1994 by Dr. Peter Greenberg in *Vibrio fischeri*. The QS system is a chemical mediated cell-to-cell communication that can regulate gene expression and the activity of the group in communities ⁴⁷. There are

many activities depending on the QS system such as production, secretion, and
detection of small signaling molecules named Autoinducers (AIs) ⁴⁸.

The presences of *Iasl* gene in *Acinetobacter* isolates were investigated by PCR.
The results indicated that 60 (49.6 %) out of 121 isolates were positive for *Iasl* gene
(Table 3). Bacteria use QS to regulate genes expression, facilitate pathogenic invasion
and spread virulence factors ⁴⁹. The QS controls local bacteria population and cell
density, which make the bacteria behave as a collaborative community such as
multicellular organism⁵⁰. Bacterial QS regulates bioluminescence, competence,
antibiotic production and secretion of virulence factors ⁵¹. This affects the formation of
biofilm ⁵²⁻⁵³, drug sensitivity ⁵⁴ and bacterial virulence ⁵⁵.
The *lasI* gene is as a part of QS system, the product of this gene being N-(3-oxo-
dodecanoyl) -L- homoserine lactone (3-oxo-C12-AHL), which interacts with *LasR* and
activates target promoters ⁵⁶. Only the multimeric form of this protein is active and can
bind to target DNA and regulate the transcription of multiple genes at high cell densities
⁵⁷.

Conclusion

MDR *A. baumannii* is considered to be a serious threat. The current study showed that
there is a high prevalence of colistin resistance in *A. baumannii* strains isolated from
Iraq. This is associated with the ability of this pathogen to acquire new genetic material
leading to increase the resistance. In addition, integrons showed a major role in
extending the bacterial ability to grow in different challenge conditions because it
allows *A. baumannii* to capture additional genetic material from other species. This
leads to the distribution and increased the resistance of *A. baumannii*. This resistance
can transform in over the world by natural transformation. The presence of a large
percentage of colistin resistant *A. baumannii* strains in Baghdad makes it urgent to avoid
unnecessary clinical use of colistin.

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Table 1: The PCR primers used and the amplicon size of different genes involved in this study

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Gene	Sequence	TM (°C)	Products size (bp)
<i>16srRNA</i>	5'-TTTAAGCGAGGAGGAGG-3' 5'-ATTCTACCATCCTCTCCC-3'	58	240
<i>mcr-1</i>	5'-CACTTATGGCACGGTCTATGA-3' 5'-CCCAAACCAATGATACGCAT-3'	59	956
<i>mcr-2</i>	5'-TGGTACAGCCCCCTTATT-3' 5'-GCTTGAGATTGGGTTATGA-3'	57	1,617
<i>mcr-3</i>	5-TTGGCACTGTATTTTGCATTT-3 5-TTAACGAAATTGGCTGGAACA-3	50	542
<i>intI2</i>	5'-CAC GGA TAT GCGACA AAA AGG-3' 5'-TGTA GCA AAC GAGTGA CGA AAT G-3'	60	788
<i>intI3</i>	5'-AGT GGG TGG CGAATG AGT G-3' 5'-TGT TCT TGT ATCGGC AGG TG-3'	60	600
<i>lasI</i>	5'- TCGACGAGATGGAAATCGATG-3' 5'- GCTCGATGCCGATCTTCAG-3'	59	402

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Table (2): Antibiotic resistance pattern of *A. baumannii* isolates.

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Class of antibiotics	Antibiotic tested	Resistant strains for 121 of <i>A. baumannii</i> isolates
penicillins	Ampicillin	% 100
	Amoxycillin	% 100
Monobactam	Aztereonam	%90
3rd generation cephalosporin	Cefotaxime	% 100
	Cefoperazone	%85.9
	Ceftazidine	%93
4 th generation cephalosporin	Cefepime	%96
Carbapenemes	Imipenem	%44.7
	Meropenem	%36
Polypeptide	Clindamycin	%91.6
	Colistin	%76
Aminoglycosides	Gentamicin	%79
	Amikacin	%72
Tetracyclines	Tetracycline	%30
Amphenicols	Chloramphenicol	%72
quinolones	Ciprofloxacin	%79
Combination	Amoxycillin/clavulanicacid	%96
	Trimethoprim/sulphametoxazole	%91.6

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Table (3): Frequency of genes involved in colistin resistance in *Acinetobacter baumannii* isolates. 484

PCR test	Positive result (%)	Negative result
<i>mcr-1</i>	89 (73.6%)	32 (26.4%)
<i>mcr-2</i>	78 (64.5%)	43 (35.5%)
<i>mcr-3</i>	82 (67.7%)	39 (32.2%)
<i>mcr-1</i> + <i>mcr-2</i>	74(61.1%)	47(38.8%)
<i>mcr-1</i> + <i>mcr-3</i>	77(63.6%)	44(36.3%)
<i>mcr-2</i> + <i>mcr-3</i>	69(57.02%)	52(42.9%)
<i>mcr-1</i> + <i>mcr-2</i> + <i>mcr-3</i>	66(54.5%)	55(45.4%)
<i>intI2</i>	78 (64.5%)	43 (35.5%)
<i>intI3</i>	81 (66.9%)	40 (33.1%)
<i>lasI</i>	60 (49.6%)	61 (50.4%)

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